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Abstract
Bivalve hemocyte competence has been measured by quantifying functional characteristics, including reactive oxygen intermediate (ROI) production after activation with zymosan or phorbol myristate acetate (PMA). However, untreated oyster hemocytes also produce ROI and RNI (reactive nitrogen intermediates) after bleeding even if not stimulated by zymosan or PMA. Extensive investigation of this parameter by flow cytometry showed that, in vitro, ROI/RNI production by untreated hemocytes maintained in seawater appeared to be independent of both bacterial burden in the serum and non-self particle phagocytosis. ROI/ RNI production in granulocytes was higher than in hyalinocytes and could be intensified when activated by zymosan but not by PMA. Both cell types used NADPH-oxidase- and NO-synthase-like pathways to produce these molecules; the NO-synthase pathway seemed relatively more dominant in hyalinocytes and NADPH-oxidase appeared more effective in granulocytes. These results provide new insights for interpreting the modulation of ROI/RNI production by untreated hemocytes shown by other studies, relative to environmental conditions or physiological status of the oysters.

Keywords: *Crassostrea gigas*; Hemocytes; Reactive oxygen intermediate (ROI); Reactive nitrogen intermediate (RNI); Flow cytometry; NADPHoxidase; NO-synthase

1. Introduction

Reactive oxygen intermediate (ROI) production by bivalve hemocytes after stimulation by non-self particles is involved in internal defense responses of bivalves (Cheng, 1996, 2000; Chu, 2000). Production of ROI, after activation by phorbol myristate acetate (PMA) (Nakayama and Maruyama, 1998; Arumugam et al., 2000a; Takahashi and Mori, 2000; Goedken and De Guise, 2004), or during phagocytosis of zymosan particles (Bachère et al., 1991; Le Gall et al., 1991; Bramble and Anderson, 1998; Lambert and Nicolas, 1998; Lambert et al., 2001), has been used frequently to estimate defense competence of bivalve hemocytes. Involvement of an NADPH-oxidase pathway in the production of ROI, superoxide anion and H_2O_2 by bivalve hemocytes has been shown (Noël et al., 1993; Torreilles et al., 1996; Winston et al., 1996; Arumugam et al., 2000b; Takahashi and Mori, 2000). Likewise, a nitric oxide (NO) synthase pathway leading to nitric oxide and peroxynitrite (RNI) synthesis has been suggested (Arumugam et al., 2000b; Gourdon et al., 2001; Torreilles and Romestand, 2001). Various methods have been employed to detect ROI production in hemocytes after activation by zymosan or PMA, either by using anti-aggregant solutions, such as Modified Alsevier Solution (MAS) (Bachère et al., 1991; Le Gall et al., 1991; Lambert and Nicolas, 1998; Lambert et al., 2001) or buffers containing glucose (Bramble and Anderson, 1997, 1998; Takahashi and Mori, 2000), both known as inhibitors of hemocyte ROI production (Torreilles et al., 1999). Several studies have described such ROI production in bivalve hemocytes without additional stimulation: similar levels of superoxide anion production (measured by NBT reduction) were observed in *Crassotrea virginica* hemocytes challenged with yeast and in non-challenged hemocytes (Fisher et al., 1996). Similar observations were done in *Crassostrea gigas* hemocytes by using a flow-cytometric (FCM) method based upon the intracellular oxidation of 2′,7′-dichlorofluorescin (DCFH) into green-fluorescent dichlorofluorescein (DCF) to measure production of ROI/RNI by the two main hemocyte sub-populations in oysters: granulocytes and hyalinocytes (Lambert et al., 2003). Indeed, this study showed not only that ROI/RNI production can be measured in hemocytes simply maintained in filtered sterile seawater (FSSW) after bleeding, but also that this production is higher in granulocytes than in hyalinocytes. Indeed, knowledge of the specific role and respective contribution of the two main *C. gigas* hemocyte subpopulations – granulocytes and hyalinocytes – in the synthesis of ROI/RNI, however, is very scarce.

Thus, several basic questions regarding ROI/RNI production by oyster hemocytes remained unanswered; two are addressed by the present study: 1) What are the causes of ROI and RNI production in untreated hemocytes, i.e. without additional activation? and 2) Are the pathways involved in ROI/RNI production by untreated granulocytes and hyalinocytes similar? Flow cytometry (FCM), coupled with DCFH oxidation, was

applied to test effects of various modulators on the ROI/RNI production by the two main *C. gigas* hemocyte sub-populations (granulocytes and hyalinocytes) maintained in seawater after bleeding.

2. Materials and methods

2.1. ROI/RNI production assays

2.1.1. Animals

Adult oysters (ca. 2 years old) from Brittany (France) were transported to the laboratory at the IUEM (Institut Universitaire Européen de laMer, Brittany). After arrival, animals were acclimated for at least 24 h in a flow-through seawater system at 10–13 °C temperature, 33–35 salinity, and then maintained in these conditions for a maximum of one week before hemolymph extraction.

2.1.2. Hemolymph collection

Hemolymph was withdrawn from the adductor muscle through a notch previously ground in the shell using a 1- or 2-mL plastic syringe fitted with a 25-gauge needle. Hemolymph from each oyster was transferred into an individual micro-tube held on ice. Individual hemolymph samples were examined under the microscope to eliminate samples with contaminating particles, such as gametes or tissue debris. Hemolymph from at least 5 animals was mixed to constitute each pool. Four pools from at least 20 oysters were made for each experiment, except as mentioned. Then, hemolymph was filtered through a 80-μm mesh before FCM analysis to avoid clogging of the flow cytometer by un-detected debris.

2.1.3. Reactive oxygen and nitrogen intermediate (ROI/RNI) production

The method for measuring ROI/RNI production was adapted from a previous report (Lambert et al., 2003). Briefly, sub-samples of hemolymph from each of the four pools were distributed into 5-mL polystyrene tubes (Falcon®, BD Biosciences, San Jose, CA, USA) and maintained on ice. A solution of 2′7′-dichlorofluorescin diacetate (DCFH-DA, Sigma) was added to yield a final concentration of 10 μM.

Flow-cytometric measurement, using a BD BioSciences FACSCalibur flow cytometer, with 0.2-μm filtered PBS azide as sheath fluid (NaCl 0.14 M, KH_2PO_4 anhydrous 10 mM, Na_2HPO_4 anhydrous 56.8 mM, di-sodium EDTA 3.4 mM, NaN_3 1.54 mM), was then performed at t=0, 60, 120 and 180 min. Between measurements, the tubes were maintained at room temperature (20–22 °C). Flow-cytometric measures of DCF green fluorescence, related quantitatively to hemocyte ROI/RNI production, at each incubation time for each hemocyte sub-population (small agranulocytes, hyalinocytes and granulocytes), were recorded on the FL1 detector (500– 530 nm, yellow-green fluorescence) and expressed as arbitrary units (method described in Lambert et al., 2003). DCFH is oxidized to fluorescent DCF by ROIs, including H_2O_2 (Bass et al., 1983) and superoxide anion but also nitric oxide (NO) (Rao et al., 1992; Curtin et al., 2002). To simplify presentation, except if noted, only results for hyalinocytes and granulocytes obtained after 120 min of incubation are presented. 2.1.4. Toxicity The potential toxicity of solvents or modulators used was tested by evaluating hemocyte viability in an antiaggregant solution (AASH) developed for bivalve hemocytes (Auffret and Oubella, 1994), at a 1/1 vol/vol dilution with hemolymph after 180 min incubation at room temperature. Viability of *C. gigas* hemocytes was evaluated by FCM, using a double staining procedure (SYBR® Green and propidium iodide, PI, Sigma). AASH was used to reduce the aggregation of cells which can lead to an over-estimation of the number of dead cells, as aggregates containing at least one dead cell (PI-stained) surrounded by numerous viable cells were counted as “dead”. AASH has been shown to have no effect on *C. gigas* hemocyte viability (data not shown). Results are presented as percentages of dead hemocytes after 180 min incubation with appropriate concentrations of modulator (Cf. Table 1: IAA, NMMA, DPI, PMA) and compared to seawater controls. 2.1.5. ROI/RNI production by untreated hemocytes The level of ROI/RNI production in untreated granulocytes and hyalinocytes was compared after 120 min incubation at room temperature in FSSW, from 5 separate experiments.

2.1.6. Bacterial burden

To explore the possible effect of bacteria present in the hemolymph on the observed ROI/RNI production in hemocytes when maintained in seawater, the possible relationship between bacterial burden in serum and hemocyte ROI/RNI production, after 120 min of incubation in seawater, was explored. Twenty oysters were bled

individually, and a tetrazolium dye reduction assay was applied to serum (after hemocytes were removed by centrifugation), to estimate bacterial cell concentration. The assay used was modified from previous methods (Volety et al., 1999). Results are expressed in units of optical density at 492 nm, which detects the enzymatic reduction of the tetrazolium dye by living bacterial cells, providing an estimate of the concentration of live bacteria. In parallel, granulocyte and hyalinocyte ROI/RNI production was evaluated as described above but for individual oysters rather than hemolymph pools.

2.1.7. Total hemocyte counts

Hemocyte concentrations in pools were evaluated to adjust the ratios of hemocytes/zymosan particles when needed. One 100-µL sub-sample from each pool was fixed by adding a formalin solution of 4% in FSSW. After 30 min incubation at room temperature (20– 22 °C) with the SYBR® Green (10× final concentrations), samples were then analyzed on the flow cytometer. A density plot visualization of side scatter (SSC) vs. FL1 permitted differentiation and gating of hemocytes stained by SYBR® Green from other particles in the hemolymph. The flow rate of the cytometer was measured for each experiment as described previously (Marie et al., 1999): briefly, a tube containing distilled water was weighed before and after a 10-min flow cytometer run to determine the volume analyzed over a known time in µL min^{-1}; this value was used to calculate the hemocyte concentration in cells mL^{-1}.

2.1.8. Hemocyte response to modulators

Granulocyte and hyalinocyte ROI/RNI production was measured after 120 min incubation with the modulators (activators or inhibitors) presented in Table 1 and also with the addition of both zymosan and cytochalasin B.

2.1.9. Statistical analysis

Significant differences between conditions during each assay were tested, using the STATGRAPHICS Plus 5.1 software, by ANOVA, or the Kruskall–Wallis test in cases wherein variance was not normal. The method used to discriminate differences between means was Fisher's least significant difference (LSD) procedure. Differences were considered significant at $p<0.05$. Possible relationships between bacterial burden and ROI/RNI production in granulocytes or in hyalinocytes were tested with the STATGRAPHICS Plus 5.1 software by calculating the R^2 value for linear and polynomial regression functions relating these two variables.

Table 1
List and concentration of modulators tested on ROI/RNI production by untreated *C. gigas* hemocytes

Modulator	Chemicals	Final concentration	Activity	Ref.
Activator	Zymosan	20 particles $hemocyte^{-1}$	Hemocyte phagocytosis, ROI/RNI production	1, 2, 3, 4, 5, 6
	Phorbol myristate acetate (PMA)	1 and 10 µg mL^{-1}	Activation of the NADPH-oxidase complex	7, 8
Inhibitor	Diphenylene iodonium chloride (DPI)	5 and 50 µM	NADPH-oxidase and NO-synthase inhibitor	9, 10, 11
	Iodoacetamide acetate salt (IAA)	1 and 10 mM	NADPH-oxidase inhibitor	12
	NG MonoMethyl-L-Arginine monoacetate (NMMA)	50 and 500 µM	NO-synthase inhibitor	13
	Cytochalasin B	10 µg mL^{-1}	Phagocytosis inhibitor	1, 2

1: Bachère et al. (1991); 2: Le Gall et al. (1991); 3: Bramble and Anderson (1998); 4: Lambert and Nicolas (1998); 5: Lambert et al. (2001); 6: Lambert et al. (2003); 7: Torreilles et al. (1996); 8: Li et al. (2000); 9: Bramble and Anderson (1997); 10: Torreilles and Romestand (2001); 11: Li et al. (2003); 12: Pipe (1992); 13: Arumugam et al. (2000a).

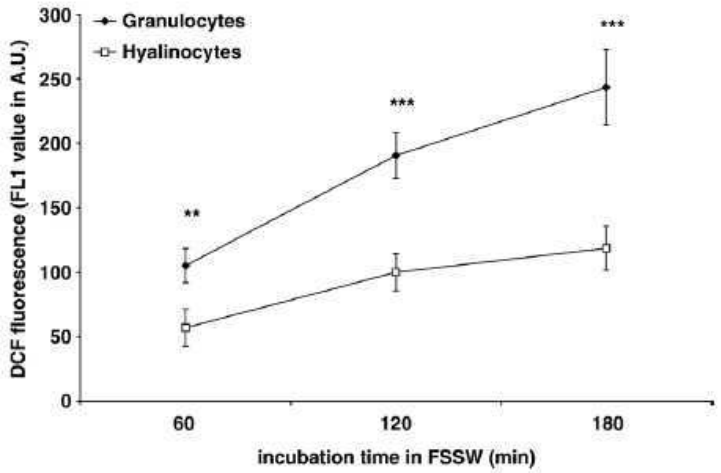

Fig. 1. Comparison of the mean level of ROI/RNI production expressed as DCF fluorescence (FL1 value, in arbitrary unit A.U., n=19+SE) in *C. gigas* granulocytes and hyalinocytes at different incubation times (60, 120, 180 min) in FSSW at ambient temperature (Mean comparison: ANOVA; NS: no significant differences, **significant differences at $p<0.01$, ***$p<0.001$).

3. Results

3.1. ROI/RNI production assays

3.1.1. Toxicity

Across all assays, mean percent mortality of hemocytes maintained for 180 min in FSSW ranged from 1.8 to 7.2%. No significant differences were observed for hemocytes exposed to the following treatments: IAA 1 and 10 mM, NMMA 50 and 500 μM, DPI 5 and 50 μM, PMA 1 and 10 μg mL^{-1}, supporting the conclusion that these chemicals did not affect hemocyte viability under these conditions.

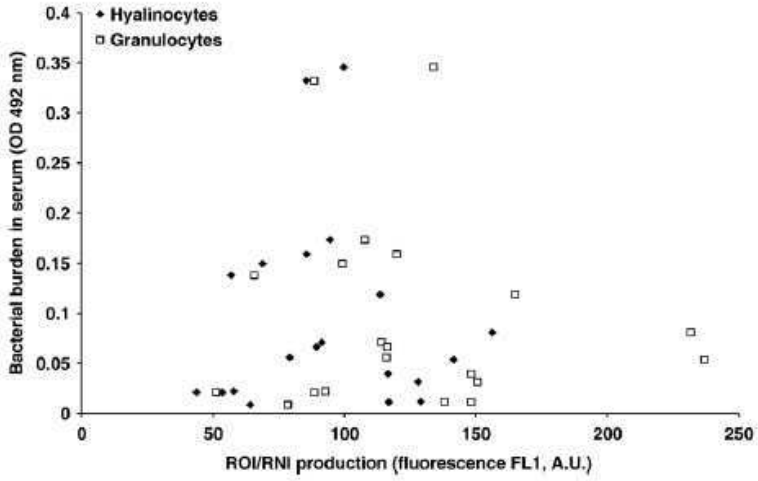

Fig. 2. Individual level of ROI/RNI production, expressed as DCF fluorescence (FL1 value, in arbitrary unit A.U.), by untreated granulocytes and hyalinocytes from twenty individual *C. gigas* after 120 min incubation at ambient temperature in FSSW, related to the bacterial burden in serum evaluated by tetrazolium dye reduction assay ($OD_{492\ nm}$).

3.1.2. ROI/RNI production in untreated hemocytes

As shown in Fig. 1, from 60 to 180 min, increases in intracellular fluorescence indicated production of ROI/ RNI. The maximal increase in ROI/RNI (slope) was found during the first 60 min, but the increase continued during the second time period. Hyalinocytes showed a significantly lower ROI/RNI production than granulocytes for all incubation times in FSSW (ANOVA, $p<0.05$).

Table 2
Mean ROI/RNI (reactive oxygen/nitrogen intermediates) production (DCF fluorescence, FL1 value, in arbitrary unit, $n=4$, except for zymosan assay $n=19$, ±standard error, SE) of *C. gigas* hemocytes (granulocytes and hyalinocytes) after 120 min incubation in filtered, sterile seawater (untreated hemocytes = control) or with various modulators

	Granulocytes (mean± SE)	Hyalinocytes (mean ± SE)
Control (untreated)	190 ± 18	100 ± 13
+Zymosan	275 ± 25*A	106 ± 11
Control (untreated)	210 ± 28	107 ± 11
+PMA 1 μg mL^{-1}	167 ± 14	83 ± 10
+PMA 10 μg mL^{-1}	138 ± 13*A	69 ± 8*A
Control (untreated)	276 ± 51	165 ± 40
+DPI 5 μM	105 ± 19*KW	46 ± 7*KW
+DPI 50 μM	49 ± 11*KW	26 ± 5*KW
Control (untreated)	162 ± 28	82 ± 31
+IAA 1 mM	51 ± 11*A	29 ± 12
+IAA 10 mM	47 ± 15*A	36 ± 13
Control (untreated)	227 ± 21	125 ± 8
+NMMA 50 μM	190 ± 25	91 ± 10*A
+NMMA 500 μM	193 ± 20	84 ± 6*A

The table combines results obtained from different experiments (*: indicate significant difference with the appropriate control, ANOVA (A) or Kruskall–Wallis (KW), $p<0.05$).
FSSW: filtered sterile seawater.
Zymosan: 20 particles $hemocyte^{-1}$.
PMA: phorbol myristate acetate.
DPI: diphenylene iodonium chloride.
NMMA: NG MonoMethyl-L-Arginine monoacetate.
IAA: iodoacetamide acetate salt.

3.1.3. Bacterial burden

Bacteria were found at various concentrations in individual oyster serum pools, as indicated by tetrazolium dye reduction assay, from 0.01 to 0.35 unit $OD_{492\ nm}$ (Fig. 2). The R_2 values for all regression functions tested relating hyalinocyte or granulocyte ROI/RNI production and serum bacterial burden were consistently smaller than 0.1 (p=0.71 to 0.98).

3.1.4. Hemocyte response to activators

3.1.4.1. Zymosan.

ROI/RNI production, measured in both granulocytes and hyalinocytes during phagocytosis of zymosan particles, is presented in Table 2. It is clear that granulocytes were stimulated to produce extra ROI/ RNI when challenged with zymosan, as compared to the level obtained with untreated granulocytes (ANOVA, p<0.01,

n=19). By contrast, no significant zymosaninduced modification in ROI/RNI production by hyalinocytes was observed.

3.1.4.2. PMA.

Addition of PMA at 1 µg mL- 1 did not modify the ROI/RNI production of either granulocytes or hyalinocytes, as compared to the level observed in untreated hemocytes (Table 2). However, when added at 10 µg mL- 1, PMA significantly reduced ROI/RNI production in all hemocyte types.

3.1.5. Hemocyte response to inhibitors

3.1.5.1. DPI.

ROI/RNI production was 62% and 72% lower with DPI at 5 µM and 82% and 84% lower with DPI at 50 µM, for granulocytes and hyalinocytes, respectively, compared to hemocytes without DPI (Table 2).

3.1.5.2. IAA.

For hyalinocytes, no significant differences were observed in ROI/RNI production with or without IAA (Table 2), regardless of dose (1 or 10 mM final concentration). However, production of ROI/RNI by granulocytes was significantly lowered after 120 min incubation in IAA, decreasing 70%, with both IAA doses (1 and 10 mM).

3.1.5.3. NMMA.

For hyalinocytes, production of ROI/ RNI was significantly lower in the presence of NMMA (50 and 500 µM: ANOVA, pb0.05), decreasing 27% and 33%, respectively compared to hyalinocytes without NMMA. For granulocytes, the addition of NMMA did not cause any significant modification of ROI/RNI production (Table 2).

3.1.5.4. Cyt B.

In the presence of cytochalasin B at 10 µg mL- 1, no significant modification of ROI/RNI production was observed for granulocytes, compared to granulocytes without cyt B (Fig. 3). The same result was obtained with hyalinocytes.

3.1.5.5. Zymosan+cyt B.

Addition of zymosan, as shown previously, increased the ROI/RNI production in granulocytes, but combined with cyt B, ROI/RNI production was reduced to the level observed in the control (Fig. 3). The same result was obtained with hyalinocytes.

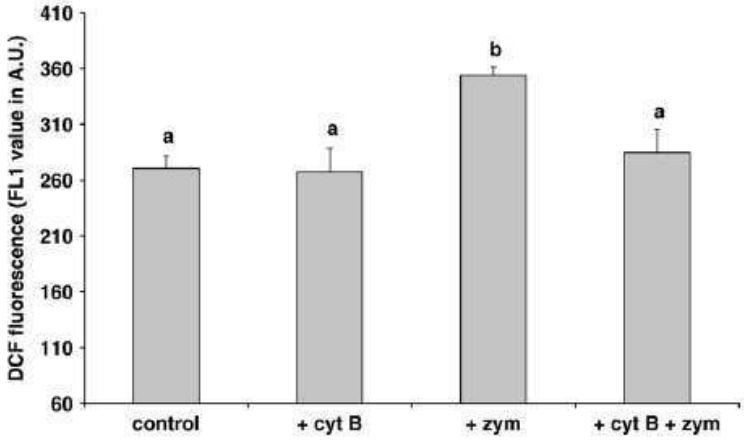

Fig. 3. Comparison of the mean level of ROI/RNI production expressed as DCF fluorescence (FL1 value, in arbitrary unit A.U., $n=4+$ SE) in *C. gigas* granulocytes at 120 min incubation at ambient temperature in FSSW (control) or after addition of cytochalasin B (+cyt B: 10 µg mL^{-1} final concentration), zymosan (+zym: 20 particles $hemocyte^{-1}$) or both chemicals (+cyt B +zym) (letters indicate significant differences between conditions, ANOVA, $p<0.05$).

4. Discussion

Recent observations (Lambert et al., 2003) revealed that oyster hemocytes produce ROI/RNI, even when simply maintained in sterile seawater after bleeding, and also that granulocytes produce more ROI/RNI than hyalinocytes. These observations prompted two basic questions regarding ROI/RNI production by oyster hemocytes: 1)What are the causes of ROI and RNI production in untreated hemocytes, i.e. without additional activation? and 2) Are the pathways involved in ROI/RNI production by granulocytes and hyalinocytes similar? The present study aimed to answer these questions.

Concerning the first question, the initial indication of the causes of ROI and RNI production in untreated hemocyteswas obtained by usingDPI, a strong inhibitor of both NADPH-oxidase and NO-synthase pathways. Approximately 80 to 85% of the fluorescence measured on untreated *C. gigas* hemocyteswas attributable to ROI/RNI produced by enzymatic systems usually described during the "respiratory burst" phenomenon. The 15 to 20% remaining is thought to correspond to a normal, constitutive physiological production of oxidant molecules. Cell metabolism, particularly within mitochondria, is known to produce ROI (Poderoso et al., 1996; Valdez et al., 2000; Marchetti et al., 2002; Cadenas, 2004), and it is likely that these ROIs associated with normal mitochondrialmetabolism could oxidize the intracellularDCFH into fluorescent DCF. For the main portion of the fluorescence measured, several hypotheses can be proposed. First, the presence of bacteria in oyster hemolymph could stimulate hemocyte ROI/RNI production following phagocytosis of these non-self particles. Indeed, bacteria can be present in hemolymph, even in healthy marine invertebrates (Sizemore et al., 1975; Tubiash et al., 1975), and especially in oysters (Olafsen et al., 1992; Garnier et al., 2007). However, in the present study, no relationship was found between bacterial burden in the serum and ROI/RNI production. Moreover, cytochalasin B did not modify the ROI/RNI production in untreated hemocytes, while it did significantly reduce the extra ROI/RNI induced by phagocytosis of zymosan. Cytochalasin B is known to inhibit actin polymerization implicated in phagocytosis (Athlin et al., 1988). These two results suggest that the ROI/RNI production by untreated *C. gigas* hemocyteswas not directly or simply linked to phagocytosis of bacteria and/or other non-self material in the hemolymph.

A second hypothesis to explain the ROI/RNI production of untreated hemocytes considers the possible stimulation of the hemocyte membrane following contact of cells with non-self materials used in experimental protocols (e.g., needle, syringe, plastic tubes), as observed for hemocytes of mussels (Pipe, 1992) and hypothesized for crustacean hemocytes (Bachere et al., 1995;Moss and Allam, 2006). However, recent studies have shown that ROI/RNI production by untreated hemocytes from marine invertebrates can also be modulated by various environmental factors experienced by the animals before hemolymph sampling, such as food availability or quality in *C. gigas* (Delaporte et al., 2006a,b), in vitro and in vivo infection by *Vibrio aestuarianus* in *C. gigas* (Labreuche et al., 2006a,b) or level of dissolved oxygen in rearing sites in lobster Homarus americanus (Moss and Allam, 2006). In-depth comprehension of such a modulation is still at an early stage, but it seems prudent to include ROI/RNI production by untreated hemocytes as a possible indicator of oyster fitness.

Addressing question 2, we also aimed to further understand the specific roles of hyalinocytes and granulocytes in ROI/RNI production when untreated. As a first result, confirming previous studies (Delaporte et al., 2003; Lambert et al., 2003), PMA did not show any capacity to modify ROI/RNI production in either untreated *C. gigas* hemocyte type, granulocytes or hyalinocytes. However, granulocytes, were shown to produce appreciably higher (approximately twice) levels of ROI/RNI than hyalinocytes. When activated by phagocytosis of zymosan particles, only granulocytes were able to produce extra ROI/RNI, at least in a systematically significant manner. These two results could indicate that the two main *C. gigas* hemocyte subpopulations possess different capabilities for ROI/RNI response. In the same manner, differences were also observed in *C. virginica*: ROI/RNI production in granulocytes and intermediate cells can be activated (PMA), but hyalinocytes cannot (Goedken and De Guise, 2004). Consequently, the proportions as well as the concentrations of hemocyte sub-populations may be considered of major importance to evaluate the functional capacity of hemocytes.

We also tested specific ROI/RNI inhibitors to better understand the respective roles of each pathway in the ROI/RNI synthesis of the two hemocyte types. Contrary to DPI findings, the two inhibitors tested had different effects on the granulocyte and hyalinocyte sub-populations. The IAA drastically reduced granulocyte ROI/RNI production (71%), but had no significant effect on hyalinocytes. By contrast, NMMA was a more potent inhibitor of hyalinocyte ROI/RNI production (27 to 33% decreases) but had no significant effect on granulocytes. To the best of our knowledge, this is the first report of different responses to an inhibitor of different hemocyte cell types; we show preferential activity of the NADPHoxidase pathway in granulocyte ROI synthesis, but the NO-synthase pathway is dominant in hyalinocyte RNI production in C. gigas. The contribution of a NOsynthase pathway to "phagocytosis-associated ROI production" in *C. gigas* total hemocytes has been

previously proposed (Nakayama and Maruyama, 1998; Torreilles and Romestand, 2001), but the present study demonstrates clearly that the NO-synthase pathway is more active in hyalinocytes than in granulocytes. Some other functional differences between *C. gigas* hemocyte cell types have been suggested recently (Terahara et al., 2006), indicating that *C. gigas* hyalinocyte phagocytosis is regulated by an integrin-dependent mechanism, but in granulocytes, phagocytosis is stimulated by other functional receptors. All these results highlight the specificity of the two main hemocyte cell types in C. gigas, according to their functional capacities and may provide new insights into the interpretation of the modulation of the hemocyte parameters by environmental conditions or physiological status of the oysters.

In conclusion, this study provided a better understanding of ROI/RNI production in untreated *C. gigas* hemocytes, measured in vitro in seawater after bleeding. This ROI/RNI production has been shown to be independent of bacterial burden in the serum and of phagocytosis of non-self particles. The two main *C. gigas* hemocyte cell types, granulocytes and hyalinocytes, have been shown to possess different ROI/RNI production capacities, granulocytes being more active in terms of constitutive production and capacity to produce extra ROI/ RNI during phagocytosis of zymosan particles. Both cell types possessed NADPH-oxidase- and NO-synthase-like pathways to produce ROI/RNI but the NO-synthase pathway seemedmore dominant in hyalinocytes; whereas, NADPH-oxidasewasmore active in granulocytes. Finally, further studies of hemocyte sub-population activities will likely lead to a better understanding of the impacts of environmental conditions on oyster fitness.

Acknowledgements

This work was supported by the MOREST national project funded by Ifremer and by the Région Basse-Normandie, Bretagne, Pays de la Loire, Poitou-Charentes and the Conseil Général du Calvados (contract Ifremer/LEMAR, IF 2003.2.2500015). Suggestions and comments of Dr Gary Wikfors, Hélène Hégaret and Dr Mirella da Silva Scuarda on the original version of the manuscript were greatly appreciated. This is contribution no. 1032 of the IUEM, European Institute for Marine Studies (Brest, France).

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